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**Application to NIH OBA to Lower Containment
for the Cloning of Full-length cDNAs of
RG4 ssns ⁽⁻⁾ RNA Virus Genomes in
Non-Pathogenic Strains of *E. Coli*.**

PI: [REDACTED]

Section 1: Statement of Work and Justification

State concisely the overall objectives and rationale of the proposed study. Include information to justify the need for cloning full-length cDNA genome copies of RG4 ssns RNA virus(es) (e.g. that such work is necessary because the investigator intends to use the cDNA genome copies to rescue infectious virus in a BL4 facility).

The scope of this work is to generate cDNA clones containing the full-length genomes of Risk Group 4 (RG4) nonsegmented negative-sense RNA viruses of the order *Mononegavirales*, including filoviruses (Marburg and Ebola virus) and henipaviruses (Nipah and Hendra virus). These constructs will be used to insert mutations in viral genes of interest and analyze the impact of these mutations on different aspects of the viral replication cycle. For rescue of the recombinant viruses, the plasmids containing the full-length viral genomes will be shipped to off-site BSL-4 facilities approved for recombinant BSL-4 work, including the NIH/NIAID Rocky Mountain Laboratories, Hamilton, MT and the NIH/NIAID Integrated Research facilities, Frederick, MD. **Transfection of cells and virus rescue will not be done at Boston University.**

The work described here was reviewed by CDC DSAT and it was confirmed that it does not fall under select agent regulation. A copy of CDC-DSAT's response is included.

We will initiate our work at Boston University's (BU) National Emerging Infectious Diseases Laboratories (NEIDL), Full-Length cDNA Laboratory (FLCL) by generating and storing plasmids containing full-length genomes of Marburg and Ebola viruses. We previously generated plasmids containing the Marburg or Ebola virus (*Zaire ebolavirus*) genome at off-site facilities (RML, University of Marburg, Marburg, Germany). Once we have the permission to handle these plasmids at BU, we will have the constructs generated at the off-site facilities shipped to BU. We will use these constructs to add mutations in specific genes to analyze the function of these genes in the context of viral infection. E.g., one planned project deals with the VP40 protein of Marburg virus which has been shown to block interferon signaling. We also aim to analyze how mutations in VP40 affect the ability of Marburg virus to infect cells derived from different animal species (human, bat, mouse). We will also generate full-length genome plasmids for different ebolavirus species (Reston, Sudan) and different isolates (e.g., *Zaire ebolavirus*, Makona isolate).

Section 2: Description of the Biological System(s) and Experimental Manipulations

Describe all biological reagents (e.g. plasmids, cell lines, prokaryotic hosts) that will be used in the experiment.

The work proposed here includes generation, manipulation, storage and shipment of DNA plasmids containing full-length viral genomes. cDNA fragments spanning the respective viral genome will be inserted into an expression plasmid under the control of the T7 RNA polymerase promoter. The expression plasmids containing the full-length clones are standard high- or low-copy plasmids containing an antibiotic resistance gene to allow selection of transformed bacteria. These plasmids are widely used in the field. Vector backbones include but are not restricted to vector 2.0 (4, 5), pBluescript (3) and p15A-Zeo (1). Amplification of plasmids

containing the viral genome sequences will be performed in E.coli K12 bacterial strains (e.g., XL1-Blue, DH10-beta, DH5-alpha). We will not perform any work in eukaryotic cells. Expression of the viral genome depends on the expression of the T7 RNA polymerase in cells. We will not use prokaryotic or eukaryotic cells expressing the T7 RNA polymerase in the FLCL in which the plasmids containing the viral genomes will be handled and stored. We will not store or handle T7 RNA polymerase expression plasmids in the FLCL.

[Describe the types of experiments to be performed.](#)

We will perform standard DNA cloning procedures. This includes amplification of fragments of the viral genomes by reverse transcriptase PCR, ligation of DNA fragments into plasmids containing the full-length viral genomes (or parts of those), *in vitro* mutagenesis, and PCR fragment replacement. DNA fragments and plasmids will be analyzed by agarose gel electrophoresis. Plasmids containing full-length viral genomes will be used to transform E.coli K12 bacteria. Transformed bacteria will be amplified (less than 500 ml) and plasmid DNA will be isolated from bacteria. Plasmid DNA containing full-length viral genomes and glycerol stocks of transformed bacteria will be handled and stored in the FLCL which is separated from the regular BSL-2 laboratory. Plasmids containing full-length viral genomes will only be removed from the FLCL to be shipped to off-site BSL-4 facilities for viral rescue.

[Identify all essential biological reagents required for the rescue of infectious recombinant RG 4 virus.](#)

T7 RNA polymerase - All viral genomes will be cloned under the control of the T7 RNA polymerase promoter within the plasmids and can only be transcribed upon addition of the T7 RNA polymerase. **Plasmids containing the T7 RNA polymerase or cells expressing the T7 RNA polymerase will be strictly physically separated from the plasmids containing full-length viral genomes.**

The RNA genomes of the nonsegmented negative-sense (NNS) RNA viruses are complementary to viral mRNA and non-infectious. Transfection of any prokaryotic or eukaryotic cells with viral NNS RNA genomes does not lead to virus production (2). Cellular RNA polymerases are not able to use viral RNA genomes as templates for transcription. All negative-sense RNA viruses encode their own RNA-dependent RNA polymerase complex. The RNA genomes are only accepted as a template for transcription and replication by the viral RNA polymerase complex when they are encapsidated by the viral nucleoprotein. Naked viral RNA cannot be replicated or transcribed and is non-infectious. To rescue negative-sense RNA viruses from plasmid DNA, all viral protein components required for replication and transcription of the viral RNA genomes need to be added. These include the proteins forming the viral RNA polymerase complex and the nucleoprotein (see below for the respective viruses). Without these support proteins and the T7 RNA polymerase, the viral genomes cannot be rescued from cDNA clones. **Support plasmids encoding viral proteins required for virus rescue will be strictly physically separated from the full-length cDNA clones. This will be achieved by using a separate laboratory to generate and store the full-length clones.**

To rescue infectious NNS RNA viruses, eukaryotic cells are transfected with plasmids encoding the full-length viral genome along with plasmids encoding the viral support plasmids. Transfection will be done in a BSL-4 facility which has the permission to do this kind of work. The T7 RNA polymerase is either constitutively expressed in the eukaryotic cells (e.g., BSR-T7/5 cells, a hamster cell line expressing the T7 RNA polymerase) or encoded by a DNA plasmid which is added to the transfection mixture. If only one of these components is omitted, virus rescue cannot take place. As mentioned above, the plasmids containing full-length viral genomes will be strictly separated from viral support plasmids, from plasmids encoding the T7 RNA polymerase, and from prokaryotic and eukaryotic cells expressing the T7 RNA polymerase.

Virus-specific support proteins

Filoviruses – To rescue Marburg and Ebola virus full-length genomes, 4 support proteins are required. These are the nucleoprotein (NP), the viral protein 35 (VP35; polymerase cofactor), the transcription factor VP30 and the RNA-dependent RNA polymerase (L) (3, 5).

Henipaviruses – To rescue henipaviruses, the following 3 support plasmids are required: nucleoprotein (N), phosphoprotein (P; polymerase cofactor), and the RNA-dependent RNA polymerase (L) (6).

Describe the types of recombinant DNA manipulations to be performed and the biosafety containment

We will solely do cloning work and plasmid amplification using E. coli K12 bacteria as described above. The plasmids containing cDNA copies of the full-length viral genomes will not be used for transfection of cells. The plasmids containing cDNA copies of the full-length viral genomes will be handled in the secured BSL-2 FLCL that is spatially separated from our regular BSL-2 research laboratory. This laboratory meets BSL-3 requirements (negative pressure, ante room, pass-through autoclave). Access to the FLCL is controlled by iris scan. The laboratory is equipped with a pass-through autoclave (solid waste) and a sink that allows disposal of liquid waste after bleach decontamination. The FLCL will exclusively be used for generating and handling plasmids containing full-length clones and fragments thereof. **We will not store or handle any support/rescue plasmids in the FLCL.**

All generated full-length genome plasmids and corresponding bacterial glycerol stocks will be stored in secured freezers and are accounted for using an electronic inventory control system. For viral rescue, the plasmids will be shipped to BSL-4 facilities which have the permission to rescue recombinant negative-sense RG4 viruses. This may include the NIH/NIAID Rocky Mountain Laboratories (RML) and the NIH/NIAID Integrated Research Facilities (IRF). We currently are discussing potential collaborations with IRF.

References

1. **Brauburger, K., Y. Boehmann, Y. Tsuda, T. Hoenen, J. Olejnik, M. Schümann, H. Ebihara, and E. Mühlberger.** 2014. Analysis of the highly diverse gene borders in Ebola virus reveals a distinct mechanism of transcriptional regulation. *Journal of virology* **88**:12558-12571.

2. **Ebihara, H., A. Groseth, G. Neumann, Y. Kawaoka, and H. Feldmann.** 2005. The role of reverse genetics systems in studying viral hemorrhagic fevers. *Thrombosis and haemostasis* **94**:240-253.
3. **Enterlein, S., V. Volchkov, M. Weik, L. Kolesnikova, V. Volchkova, H. D. Klenk, and E. Mühlberger.** 2006. Rescue of recombinant Marburg virus from cDNA is dependent on nucleocapsid protein VP30. *J Virol* **80**:1038-1043.
4. **Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* **69**:1011-1020.
5. **Volchkov, V. E., V. A. Volchkova, E. Mühlberger, L. V. Kolesnikova, M. Weik, O. Dolnik, and H. D. Klenk.** 2001. Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. *Science* **291**:1965-1969.
6. **Yoneda, M., V. Guillaume, F. Ikeda, Y. Sakuma, H. Sato, T. F. Wild, and C. Kai.** 2006. Establishment of a Nipah virus rescue system. *Proceedings of the National Academy of Sciences of the United States of America* **103**:16508-16513.

Section 3: Biosafety and Biosecurity Procedures

All research work involving the cloning of full-length cDNA constructs derived from RG4 viruses of the order *Mononegavirales* will be conducted in the designated Full-Length cDNA Laboratory (FLCL) space in the NEIDL.

I. FLCL Facility

The FLCL is a specifically designated BSL-2 laboratory and all personnel that require access to the facility will be approved by the Director of NEIDL.

The NEIDL FLCL facility is physically separated from the rest of the BSL-2 laboratories on the floor by walls and an entrance door that leads to the airlock. The entrance door is locked at all times, has a self-closing mechanism, and is secured by a biometric iris scanner. The facility is designed with room air pressure sensors that are installed at each door entrance, a pass-through autoclave, and foot and elbow operated sink.

The FLCL room air is designed to flow in a unidirectional manner and the facility is kept under constant negative pressure. The room air pressure monitor is connected to the Building Automation System (BAS) and is monitored at all times. The BAS monitor will alarm in the event that a room air pressure excursion occurs. The supply and exhaust air system is dedicated and is not adjoined to the other BSL-2 laboratories on the floor. The exhaust system is HEPA filtered and the filters are scanned and tested annually and decontaminated and replaced when needed.

II. Access Control

Access to the facility is designated and approved by the Director of NEIDL. All personnel who are approved to access the space are required to enroll in the security iris scan system which is administered and maintained by BU Public Safety (PS).

a) General Cleaning

Laboratory personnel approved to work in the facility perform the routine cleaning and upkeep of the space. Work surfaces are routinely cleaned with chemical disinfectant at end of their work.

b) Maintenance and Repair

All clones will be secured prior to any maintenance or repair work by authorized personnel within the facility. All work surfaces and equipment will be cleaned and disinfected.

III Security, Storage and Inventory of Clones

All clones that are used and stored in the FLCL are secured within the facility at all times. Any transfer of clones will be first reviewed and approved by the Principal Investigator (PI) before it will occur.

a) Security

All clones are secured and stored within the FLCL facility. The facility is secured by a locked door and a biometric iris scanner. Personnel who have been authorized by the Director of NEIDL, met all training requirements and presently enrolled in the iris scan system administered by the BU PS can access and enter the space.

b) Storage

All clones for long-term storage are stored in secured freezers. The freezers are located within the FLCL facility that is secured by a door that is locked at all times and entrance is through a biometric iris scanner.

c) Inventory

The authorized PI and researchers will maintain an updated electronic inventory record of clones. The inventory record will keep track and record of the following information:

1. Quantity of cDNA produced and used in the facility
2. Names of personnel that access the cDNA
3. Date cDNA are added to or removed from the inventory

The authorized PI will conduct an annual inventory check of full-length clones. Discrepancies will be reported to PS and the BU Biosafety Officer (BSO).

IV. Material Flow

All clones will be stored and used within the FLCL facility using BSL-2 practices and procedures. Personal protective equipment include laboratory coats, gloves, shoe covers and safety glasses that are used whenever performing procedures that require protection of the eyes.

a) Transfer of Clones

Any transfer of clones to another collaborating laboratory facility outside of the NEIDL FLCL will be approved by the PI. Packaging and shipping of the material will comply with the International Air Transport Authority (IATA) and the US Department of Transportation (DOT) regulations. Recipients of the clones must be authorized to receive and possess the material.

V. Inspections and Verifications

The BU BSO will conduct an annual inspection of the FLCL facility to review compliance to biosafety and biosecurity requirements. Any findings will be reported by the BSO to PI for correction.

VI. Biological Waste

All biological wastes generated within the FLCL facility will be treated as BSL-2. All solid wastes will be collected into covered waste containers lined with autoclavable bags. The bags will be autoclaved through the pass-through autoclave within the FLCL facility prior to placing in boxes for final disposal and incineration.

Liquid wastes will be treated with chemical disinfectant overnight prior to being disposed into the sanitary sewer.

Any sharps wastes generated will be collected into puncture-proof sharps containers prior to autoclaving and final disposal and rendering.

VII. Annual Reporting

The BU BSO will prepare and submit a written report to the BU Institutional Biosafety Committee (IBC) as part of the annual reporting. The report will include events and activities including personnel training status, any breach in containment, laboratory incidents, and changes in biosafety or biosecurity procedures.

Section 4: Personnel

Provide information about the personnel who will be performing the work.

- Describe the level of expertise, experience and training of personnel who will have access to full-length cDNA reagents (e.g. full-length cDNA molecules, plasmid constructs, transformed *E. coli*).

Initially, five researchers will have access to the FLCL:

- [REDACTED]. [REDACTED] is an internationally renowned expert in the field of Marburg and Ebola virus reverse genetics systems and has over 25 years of experience in working with RG4 pathogens. She was trained at the Philipps University of Marburg, Marburg, Germany by Drs. Feldmann and Klenk. She joined Dr. Feldmann's lab in 1988 as a diploma student and has worked on filoviruses and other RG4 pathogens since then. She is highly experienced in handling RG4 pathogens under BSL-4 conditions. Before she joined BU in 2008, she was Assistant Professor, PI, and group leader at the University of Marburg. [REDACTED] has extensive experience in analyzing replication and transcription of nonsegmented negative-sense RNA viruses and pioneered the establishment of reverse genetics systems for filoviruses. The first minigenome systems for both Marburg- and Ebolavirus were established in her laboratory. In addition, she contributed to the first full-length Ebola virus reverse genetics system and developed the first full-length infectious reverse genetics system for Marburg virus. Members of her laboratory generated and handled full-length cDNA clones of Marburg and Ebola viruses at the University of Marburg and at the NIH/NIAID Rocky Mountain Laboratories (RML), Hamilton, MT.

- [REDACTED]. [REDACTED] has more than 10 years of experience in rDNA work, including handling filovirus full-length clones. She joined [REDACTED] laboratory at the University of Marburg in 2003 and continued to work on filoviruses since then. Together with [REDACTED], she joined BU in 2008. [REDACTED] has extensive experience in handling RG4 pathogens. Starting in 2010, she has been granted permission to perform BSL-4 work at the NIH/NIAID Rocky Mountain Laboratories as a guest researcher. [REDACTED] has access to the RML FLCL and is experienced in handling, manipulating and inventorying filoviral full-length cDNA clones.
- [REDACTED]. [REDACTED] has more than 10 years of experience in rDNA work. He was trained in rDNA work at the Beth Israel Deaconess Medical Center, Boston, MA (2003-2004) and the University of Wisconsin-Madison (2004-2010). [REDACTED] joined [REDACTED] laboratory in 2010 as a postdoctoral fellow. He has been trained in BSL-4 work at the Texas Biomedical Research Foundation, San Antonio, TX and serves as a BSL-4 mentor at the NEIDL at BU to prepare staff for future BSL-4 work.
- [REDACTED]. [REDACTED] joined [REDACTED] laboratory in 2011 and has been trained in rDNA and BSL-2 work. [REDACTED] is highly experienced in using Marburg and Ebola virus minigenome systems. Since she works extremely carefully, she has recently been accepted for the NEIDL BSL-4 training program. [REDACTED] will be responsible for organizing and maintaining the FLCL.
- [REDACTED]. [REDACTED] joined [REDACTED] laboratory in 2015. She is co-supervised by [REDACTED], who is a world-leading expert in studying replication and transcription of negative-sense RNA viruses. [REDACTED] has been extensively trained in rDNA work in the [REDACTED] and [REDACTED] laboratories. Her research project deals with replication initiation mechanisms of Ebola virus. Due to her extraordinary lab skills and her high reliability, she is very well suited to perform work with full-length cDNA clones of RG4 pathogens.
- Describe the screening policies and procedures used to evaluate individuals who have access to full-length cDNA reagents.
 - o For security clearances other than Federal Public Trust Level 5 (Minimum Background Investigation for Federal employees) or a Federal Bureau of Investigation Security Risk Assessment (for individuals involved in Select Agent research), investigators should provide a listing of the information sources used in the preparation of the background investigation process

All personnel who will work in the FLCL complete and maintain the following evaluation requirements:

- **Suitability, Medical and Background Checks**

Personnel who work in the laboratories at the NEIDL undergo routine background checks as policy and part of the overall suitability program. Individuals who work or have access to laboratories without select agents and toxins are required to successfully complete the following requirements initially after hiring. Periodic recertification of these requirements are performed at least annually or as determined.

- o Criminal Background Check – criminal background check is completed in accordance with BUPS guidelines and all federal, state and local laws and regulations.
- o Basic Background Check – basic background check include but not limited to, verification of Social Security Number, academic credentials, past employment history, professional licenses and credentials, Sexual Offender Registry Information (SORI), credit history check, and RMV/DMV driving record.
- o Medical Clearance – medical clearance include occupational health evaluation by the BU Research Occupational Health Program (ROHP), drug screening, psychological screening, and immunization/titer level review (as determined based on job function).
- **Training requirements**

All personnel who are approved and authorized to work or access the FLCL are required to complete the following training:

 - o General Training

All personnel who work in laboratories at BU are required to complete their Laboratory Safety Training initially upon hire and annually thereafter. The training covers general laboratory safety, biosafety and blood borne pathogens.
 - o Security Awareness Training

All NEIDL Personnel receive training in identifying suspicious behavior and insider threat training. This training includes identifying indicators and making observations and instructions on making the appropriate notifications. This training occurs on an annual basis.
 - o FLCL-specific Training

Personnel who are approved and authorized to work in the FLCL facility are trained on the Biosafety and Biosecurity Plan and the Incident Response and Reporting Plans.

Section 5: Incident Response, Incident Reporting and Training

Provide information describing the institutional emergency planning and program management.

- Describe the institutional policies and procedures responsible for managing:
 - Biosafety incidents (exposures or laboratory acquired infections)
 - Biosecurity incidents (thefts or threats)
 - Hazardous materials incidents (spills or releases)
 - Policies and procedures with outside health authorities
- Describe the institutional policies or procedures involving biosafety and biosecurity training, including the frequency of the training requirements.

BU operates under the Incident Command System (ICS) in its response to incidents and emergencies in the laboratories at BU and the NEIDL. The FLCL at NEIDL falls under the

oversight and purview of this system and events involving the FLCL facility will follow the reporting, notification, communication and response established.

I. Emergency Planning and Program Management

a) Laboratory Injuries and Exposure

Personnel who are injured or exposed while working in the FLCL must immediately stop their work, take care of the injury, report the incident to the Control Center at [REDACTED], and proceed to the Research Occupational Health Program (ROHP) for medical attention.

1. Cuts, punctures, lacerations and other breaks in the skin: When an injury that causes a break in the skin occurs, the wound must be immediately washed under clean running water and disinfectant soap. The wound should be covered with a clean bandage upon washing and then proceed to normally exit the laboratory by removing the rest of the personal protective equipment and report to ROHP for medical attention.

2. Splatter to the eyes, nose, or mouth: When a direct splatter to the mucous membrane of the eyes, nose, or mouth occurs, the affected area must be flushed with copious amount of clean running water under the eyewash station. After flushing the area, proceed to exit the laboratory normally by removing the rest of the personal protective equipment and report to ROHP for medical attention.

b) Theft or Loss of Clones

Any discovery of a theft, loss, or inventory discrepancy of clones stored in long-term storage must be immediately reported to BU Public Safety (PS) at [REDACTED] and the BU BSO. PS will work with the PI and conduct a follow up investigation. The incident will be reported to the BU Institutional Biosafety Committee by the BSO.

c) Spills and Releases

Spills must be immediately cleaned up and disinfected using the biological spill kit in the FLCL. All wastes generated from the clean-up will be autoclaved prior to final disposal. Spills will be reported to the BSO.

d) Security Breach

Any discovery of a security breach or suspicious circumstances in the laboratory must be immediately reported to PS at [REDACTED] and the BSO. PS will conduct an investigation and work with the PI to determine the breach, review facility access records and iris scan system activities.

II. Policies and Procedures

a) Institutional Biosafety Committee (IBC)

All research work with biohazardous materials and/or rDNA at BU will be reviewed and approved by the BU IBC before any work can begin.

b) Boston Public Health Commission (BPHC)

Research work involving rDNA is reported by the BU IBC to the local City of Boston Public Health Commission under the permit requirement for working with rDNA.

c) Environmental Health and Safety (EH&S)

Laboratories approved to perform life science research by the IBC are inspected and reviewed by the BU EH&S.

III. Institutional Biosafety and Biosecurity Training Policies

Personnel undergo Laboratory Safety training with annual refresher and additional training as required by the nature of the research. Biosecurity training is included. Training modules are available for work that includes rDNA, blood-borne pathogens, BSL3 containment, shipping, chemical safety, controlled substances. Pathogen-specific training is provided by ROHP and the PI when appropriate. If animals are used, training modules include IACUC (Institutional Animal Care and Use Committee) Orientation with annual refresher, and AALAS training. No animals will be used in this cDNA cloning project.

Section 6: Compliance Assurance and Documentation Requirements

- An appropriate institutional official must be appointed to oversee the biosafety and biosecurity plan of the institution for all work involving full-length cDNA clones of RG4 viruses. Contact information for this individual should be provided.

██████████ is designated as the institutional official to oversee the biosafety and biosecurity plan of the institution for all work involving full-length cDNA clones of RG4 viruses.

Please contact ██████████ at: Boston University, 1 Silber Way, ██████████, Boston, MA 02215, ██████████, ██████████.

- The institutional official will be responsible for periodic re-evaluation of the biosafety and biosecurity plan.
- The institutional official will be responsible for filing an annual report with the IBC. The report will include:
 - o Any incidents of containment breaches or laboratory exposures
 - o Changes in biosafety of biosecurity procedures
 - o Documentation of on-going periodic training of personnel in all applicable procedures
- A copy of the annual report will be provided to NIH OBA for review by the RAC as required

Responsibilities of ██████████:

- Periodic re-evaluation of the biosafety and biosecurity plan.
- Filing an annual report with the IBC. The report will include: any incidents of containment breaches or laboratory exposures; changes in biosafety of biosecurity procedures; documentation of on-going periodic training of personnel in all applicable procedures.
- Provide a copy of the annual report to NIH OBA for review by the RAC as required.

